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Resveratrol for Protection against Statin Toxicity in C2C12 and H9c2 cells

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Abstract

Statins are among most commonly prescribed drugs for treatment of high blood cholesterol. Myotoxicity of statins in certain individuals is often a severe side effect leading to withdraw. Using C2C12 and H9c2 cells, both exhibiting characteristics of skeletal muscle cells, we addressed whether resveratrol (RSV) can prevent statin toxicity. Statins decreased cell viability in a dose and time-dependent manner. Among the five statins tested, atorvastatin, simvastatin, lovastatin, pravastatin and fluvastatin, simvastatin is the most toxic one. Simvastatin at 10 μ M caused about 65% loss of metabolic activity as measured by MTT assays in C2C12 cells or H9c2 cells. Inhibition of metabolic activity correlates with an increase in caspase activity. RSV was found to protect H9c2 cells from simvastatin induced activation of caspase 3/7. However, such protection was not found in C2C12 cells. This cell type dependent effect of RSV adds to the complexity in muscle cell toxicity of statins.

Introduction

Cardiovascular disease is the leading cause of death worldwide. Large-scale clinical studies have shown that lowering blood cholesterol significantly reduces coronary disease-related deaths (1,2). Statins, the inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, lower blood cholesterol by blocking mevalonate synthesis, the rate limiting step of cholesterol biosynthesis pathway in the liver. In addition to this canonical effect, recent *in vitro* and *in vivo* studies have found that statins exhibit non-canonical effects, including improving endothelial function, stabilizing atherosclerotic plaque and preventing thrombogenic events (3–5). Statins have also been found to exhibit anti-inflammatory and cardiac protective effect (6). For most people, statin drugs are safe and well tolerated. However, up to 30% of statin users reported intolerance often with muscular or joint discomfort, leading to noncompliance to the drug therapy. Statin associated muscle symptoms vary from benign myalgia or mild myopathy to myositis or fatal rhabdomyolysis (1,7,8). It has been observed that the symptoms of skeletal muscle myopathy are independent of the efficacy of statins in lowering cholesterol (9).

Mevalonate is also an intermediate for the synthesis of isoprenoids, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate. These isoprenoids are involved in protein

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to 100x concentration in phosphate-buffered saline (PBS) prior to adding to cells for the final concentration of 1x in culture. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO). Ac-DEVD-AMC was obtained from Cayman Chemical Company (Ann Arbor, MI).

Cell Culture

C2C12 murine myoblasts were a generous gift from Dr. Donna Zhang's Lab. H9c2 rat myoblasts were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained by weekly subculture in 100 mm dishes (Genesee Scientific, San Diego, CA) in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Grand Island, NY) supplemented with heat-inactivated 10% fetal bovine serum (FBS, Omega Scientific, Tarzana, CA), containing 25 mM glucose, 1 mM sodium pyruvate, 2 mM L-glutamine and 100 unit/ml penicillin/streptomycin. Upon reaching 70–80% confluency, cells were subcultured for stock, seeded into 100 mm dishes for Western blot experiments, or into 6-well or 24-well plates for cytotoxicity assays. Cells were treated with various doses of statins at 24 hours after plating when the density reached 80% confluency. Resveratrol was added to cells 1 hour before statin treatment.

MTT Assay

Cells were seeded at a density of 3×10^4 per well in a 24-well plate containing 1 mL culture medium. After 24 hours of seeding, statins were added for 24 hour treatment and at the end of the treatment, MTT was added to the culture media to a final concentration of 0.16 mg/mL. Following 30 min incubation in a tissue culture incubator at 37°C with 5% CO₂, purple crystals of reduced formazan were observed inside cells under a phase contrast microscope. The culture medium was removed and the formazan crystals were dissolved in isopropanol containing 4 mM HCL and 0.1% NP-40. Cell viability was calculated by comparing the absorbance at 570 nm subtracted from the background reading at 690 nm of treated cells to that of untreated cells, which were considered as 100% viable.

Morphology and Cell Number Counting

Cells were grown on 6-well plates or 100 mm dishes. Following incubation with simvastatin with or without RSV, the morphology of cells was recorded under a phase contrast microscope (EVOS FL, Life Technologies) with 4x sitting. The percentage of viable cells was determined by counting cells from randomly selected 3 fields from each sample.

Annexin V Staining

Cells were seeded onto cover glasses placed in a 24 well plate at a density of 3×10^4 cells/mL/well. At 20–24 hours after seeding, cells were incubated with simvastatin with or without RSV for 16–20 h. After washing with PBS, 20 µL of Annexin V-Fluos (Roche Life Science) was added in 100 µL of Annexin V binding buffer to each well for 15 min incubation at room temperature in the dark. Cell samples were washed briefly with PBS before fixation in 2% formalin for 10 min at room temperature. After washing with PBS, the cover glass was mounted on a microscope slide for examination and recording images under Olympus BX53 fluorescent microscope with attached digital camera.

Caspase Activity Assay

At 16 hr after statin treatment, detached cells from 6-well plates were collected by centrifugation at 3000 RPM for 3 minutes and washed once with PBS before combining with the adherent cells from the same well. Cells were scraped off the well in 60 μ L of lysis buffer (0.5% Nonidet P-40, 0.5 mM EDTA, 150 mM NaCl, and 50 mM Tris pH 7.5). Caspase 3/7 activity was measured using a fluorogenic substrate Acetyl Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC). Cell lysates (50 μ L/well) were incubated at 37°C for 1 h with a master mix containing 10 μ M of synthetic peptide substrate Ac-DEVD-AMC in a total volume of 200 μ L in a 96-well plate. Caspase-3/7 activity was expressed as fluorescent intensity unit detected by a 96-well fluorescence plate reader (BioTek Synergy 2, Winnski, VT) with an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

Western Blot Analysis

Cells were washed twice with ice cold PBS for protein extraction using RIPA buffer [(50 mM Tris-HCL pH 7.4, 1% NP-40, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF with freshly added protease inhibitor cocktails tablets (Fisher Scientific, Pittsburgh, PA)]. After removing insoluble cell debris by centrifugation at 12,000 \times *g* for 10 min, protein concentration in the supernatant was determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). An equal amount of proteins from different samples were boiled in SDS buffer and loaded for separation based on molecular weight by electrophoresis using 12% polyacrylamide gel. Separated proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules CA) by electrophoresis at 100 Volts and 4 °C for 1 hour, or at 30 Volts and 4 °C for overnight. After blocking the membrane for non-specific protein binding using 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) at room temperature (RT) for 1 hour, PVDF membranes were incubated overnight at 4°C with primary antibodies (1:1,000, Rabbit polyclonal, Cell Signaling Technology, Beverly, MA) against caspase-3 or caspase-7. Vinculin was probed as an internal loading control using a polyclonal antibody (1:1,000, H300, Santa Cruz Biotechnology Inc, CA). The membranes were washed in TBST and incubated with appropriate secondary antibodies (Santa Cruz Biotechnology Inc, CA) conjugated with horseradish peroxidase for 1.5 h at room temperature. Enhanced chemiluminescence (ECL) was used to detect bound antibodies by ChemiDoc XRS+ imaging system with Image Lab software (Bio-Rad, Hercules, CA).

Statistical Analysis

All cytotoxicity experiments were performed in triplicates (treatment) and repeated at least three times. Significant differences were determined by Analysis of Variance (ANOVA) with $p < 0.05$ or student's t-test with $p < 0.01$.

Results

Cytotoxicity of Statins

Four commonly prescribed statins, Atorvastatin, Simvastatin, Lovastatin, and Pravastatin were tested for cytotoxicity, plus Fluvastatin, which has a weak activity in reducing

blood cholesterol. MTT reduction is one of the most commonly used methods for measuring metabolic viability. The yellow tetrazolium of MTT is reduced by NAD (P) H oxidoreductase in the mitochondria to purple colored formazan, allowing high throughput spectrophotometric quantification. Decreases in MTT conversion reflect the degree of metabolic inactivation of the cells.

Dose-dependent response studies were performed to define the most versus the least toxic statins in two muscle cell lines using the dose range of 0.1–10 μM . As shown in figure 1, all statins induced a dose-dependent inhibition of metabolic viability. Atorvastatin and pravastatin are least toxic in either cell line, which exhibited similar trend of dose responses (Fig 1). Although metabolic viability significantly decreased with lovastatin and fluvastatin, Simvastatin appears to be most toxic with an LC50 of 2.6 μM and 1.9 μM in C2C12 and H9c2 respectively. The LC50 value was calculated based the viability as measured by MTT assay as a percentage over control untreated plotted on the Y axis and the concentration of the statins in micromolar plotted on the X axis. A linear or logarithmic equation depending on the dose response curve obtained was used to calculate the LC50.

Since simvastatin is the most potent drug in inducing cytotoxicity among all the five statins, simvastatin was used for studying time-dependence of metabolic inhibition. Both C2C12 and H9c2 cells showed time dependent loss of viability as measured by MTT assay from 1 to 48 hours, and C2C12 cells maintained about 40% metabolic activity at 24 hours and required 48 hours for near complete elimination of metabolic viability (Fig 2A). In contrast, H9c2 cells lost most if not all metabolic activity by 24 hours (Fig 2B). Therefore, although both cells exhibited a similar trend in time dependent loss of metabolic viability, H9c2 cells showed a steeper decline after 8 hours.

Morphologic observation and cell number counting of C2C12 cells and H9c2 cells suggest that statins induced cytostasis as well as cell death (Fig 3). C2C12 cells showed a faster proliferative rate, with the number of cells doubled over 24 hours. Treatment of simvastatin for 24 hours caused about 25% loss of C2C12 cells (Fig 3). H9c2 cells is slower in proliferation, showed about 50% loss of cell number (Fig 3). In addition to inhibition of cell number increases, detached cells in statin treated groups showing shrinkage, typical morphology of apoptotic cells (Fig 3).

Using caspase -3/7 activity assay, we measured apoptosis quantitatively. A higher baseline caspase activity in C2C12 cells reflects a higher number of cells compared to H9c2 cells at the time of measurements. Nevertheless, a dose and time dependent increase of caspase activity was observed in C2C12 cells and H9c2 cells with simvastatin treatment (Fig 1K&L, 2C&D). The maximal fold of caspase activity increase is similar between C2C12 cells and H9c2 cells within each experiment of comparing the effect of simvastatin. Caspase 3/7 activity increased with the increasing dose of simvastatin from 0.1 to 1 μM and reached the highest with 1 μM in C2C12 cells and declined with higher doses (Fig 1K). The decline of caspase activity with 5 to 10 μM simvastatin may indicate a mixed mode of cell death, with high doses are expected to cause necrosis in addition to apoptosis (28). H9c2 cells showed somewhat resistance until 1 μM of simvastatin and dose dependent increases of caspase activity in 2 to 10 μM dose range (Fig 1L). The time course studies revealed that caspase

activation reached the highest at 8 hours and declined after 16 hours in C2C12 (Fig 2C). The peak of caspase activation occurred at 24 hours and decreased at 48 hours in H9c2 cells (Fig 2D). The decline of caspase after 16 hours for C2C12 cells or 24 hours for H9c2 cells indicates that the process of apoptosis occurred and finished within the period of time, and discontinued afterwards.

Resveratrol Inhibits Apoptosis in H9c2 Cells

Caspase activity assay provides a quantitative measure of apoptosis and was used to determine the effect of RSV on statin induced cytotoxicity. C2C12 or H9c2 cells were pretreated with RSV (50 μ M) for 1 h before simvastatin (5 and 10 μ M) treatment. Cells were incubated for 16 h for caspase activity assay. Interestingly, RSV inhibited caspase activation in H9c2 cells but not in C2C12 cells (Fig 4A&B). In C2C12 cells, RSV induced activation of caspase 3/7, which appeared to be additive with simvastatin (Fig 4A).

To confirm caspase activation with simvastatin and the inhibition by RSV in H9c2 cells, we measured caspase 3 and caspase 7 cleavage by Western blot. Caspase 3 protein was probed using an anti-caspase 3 antibody that recognizes the full length 32 KDa form and 17 KDa cleaved form of caspase 3. Similarly, anti-caspase 7 antibody recognized the full length 37 KDa and 20 KDa cleaved form of caspase 7. Vinculin was used as a loading control. In C2C12 cells, there is cleavage of caspase 3 and caspase 7 due to simvastatin treatment, with or without RSV pretreatment (Fig 4C). In contrast, in H9c2 cells, the cleavage of caspase 3 or caspase 7 due to simvastatin treatment was inhibited by 50 μ M RSV pretreatment (Fig 4D). Therefore, the caspase cleavage results are consistent with that of caspase activity assay, showing that RSV mediated protection against Simvastatin toxicity is specific for H9c2 cells.

Cell morphology was recorded using light microscopy to further demonstrate the effect of RSV on apoptosis induced by simvastatin (Fig 5A). Treatment with 10 μ M simvastatin resulted in condensed, rounding up and floating either C2C12 or H9c2 cells. RSV treatment reversed cell morphology to normal in H9c2 cells with fewer apoptotic cells but failed to do so in C2C12 cells (Fig 5A).

Annexin V staining was performed to confirm H9c2 cell specific inhibition of apoptosis by RSV. Annexin V is a 36 KDa protein that has a high affinity for phosphatidylserine, which normally locates in the inner side of the plasma membrane, but migrates to the outer surface of apoptotic cells where it readily binds to Annexin V. Positive Annexin V staining increased in C2C12 cells due to simvastatin treatment with or without RSV pretreatment (Fig 5B&C). However, RSV pretreatment blocked simvastatin from inducing Annexin binding in H9c2 cells (Fig 5B&C).

Apoptosis results in a loss of cell number in adherent culture. To determine cell number loss, cells were seeded on 6- well plates at 3×10^4 cells/mL and allowed to attach overnight. The next day, 3 random pictures were taken from each well. At this time point (T0), the number of cells is set to 100%. Cells were then incubated in the absence or presence of RSV (50 μ M) for 1 hour prior to incubation with 10 μ M simvastatin for 16 h-20 h. At 16 h later (T16), cell number was recorded under a phase contrast microscope. Three random visual

fields from triplicate samples were chosen and the number of adherent cells was counted using ImageJ software. The number is expressed as a % of T₀ control (Fig 5D). The number of untreated cells increased to 220% and 140% in C2C12 and H9c2 cells respectively due to continuous cell proliferation. However, with simvastatin treatment, the number declined to about 80% or 50% in C2C12 or H9c2 cells respectively. RSV alone induced loss of cell number in C2C12 cells and did not reverse simvastatin induced loss of cell number (Fig 5D). In contrast, RSV did not impair cell growth dramatically in H9c2 cells, but inhibited simvastatin from inducing loss of cell number (Fig 5D). These data added together supporting RSV protects H9c2 cells but not C2C12 cells from undergoing apoptosis.

The inhibitory effect of RSV on simvastatin induced apoptosis promoted us to determine whether RSV can block simvastatin from inhibiting metabolic viability. We conducted MTT assay in C2c12 and H9c2 cells treated with simvastatin with or without RSV pretreatment. The metabolic viability rate was reduced with simvastatin treatment at 5 or 10 µM dose in both cell lines, which was not affected by RSV pretreatment (data not shown).

Discussion

Our data indicate that statins induce cytotoxicity in H9c2 and C2C12 in a time and dose-dependent manner. We found that simvastatin is the most potent inducer of cytotoxicity, consistent with the findings from clinical observations. Such feature of simvastatin may be related to its lipophilicity, implying its ability to cross cell membrane more readily than the hydrophilic statins, e.g. pravastatin. Interestingly, RSV inhibited apoptosis only in H9c2 as shown in caspase-3/7 activity, caspase 3/7 cleavage, Annexin staining and cell morphology, but not C2C12 cells. Therefore, although C2C12 and H9c2 cells share the relevance with their characteristics of skeletal muscle cells and their response to statins, they respond to RSV differently.

RSV has been reported to inhibit apoptosis of cardiomyocytes by experimental ischemic injury (29). This is consistent with our observation of RSV inhibiting apoptosis of H9c2 cells, since this cell line was originally derived from rat myocardial tissue (27). Although the mechanism of apoptosis inhibition remains to be elucidated, RSV has been shown to decrease oxidant generation and increase antioxidant defense systems (18,30–32). However, we did not observe a protective effect against statin toxicity by N-acetylcysteine, tert-butylhydroquinone, butylated hydroxyanisole and phenylenediamine, the antioxidants have often been used for cell culture experiments (data not shown). The cell type dependent protection of RSV also points to a mechanism outside antioxidant.

The benefit of combining RSV with statin use has been tested in experimental animals related to the reported functions of RSV as an antioxidant, angiogenesis, anti-platelet, or anti-inflammatory agent. RSV improves the efficacy of statins by reducing oxidized LDL, LDL, total cholesterol, or triglycerides (33). In a clinical trial with 75 enrollments of high risk cardiovascular patients on statin treatment, 1 year supplement of RSV-rich grape product resulted in significant improvement of fibrinolytic status and decreases of inflammatory biomarkers (34). With hypercholesterolemia rats, the combination of statin plus RSV is cardiac protective against ischemic injury (29). Current pharmacologic

treatment of acute myocardial ischemia in the clinic include statins for cardiac protection (35–39). Combining RSV with statins is expected to achieve further reduction in cardiac injury. Animal experiments have also showed a role of RSV for inhibition of heart failure in the models of pressure overload, myocarditis and cardiomyopathy due to doxorubicin chemotherapy (40). These lines of evidence support the concept of combining RSV with statins for improved outcome in treatment of dyslipidemia, coronary disease and heart failure.

Statin toxicity studies in humans have revealed genetic elements in influencing individual's susceptibility. The severity of side effects appears to be affected by heritable factors as well as epigenetic factors. Genome wide association studies revealed a particular SNP in the solute carrier anion transporter family 1B1 (SLCO1B1) gene alters one amino acid in its encoded protein, a hepatic organic anion transporter OATP1B1, in patients with simvastatin-induced myopathy. This SNP contributes to an increased plasma concentration of statins (41,42). Other genetic factors affecting statin toxicity include UDP glucuronosyltransferase, which metabolizes the lactone form of statins. Statin intolerance in humans have also been linked to ryanodine receptors, leukocyte immunoglobulin-like receptor, and serotonin receptor genes HTR3B and HTR7 (1). While cell culture experiments do not replicate the intolerance aspect of adverse drug reaction, the difference in genetic background of each cell line may influence the difference in cytotoxicity response.

Abbreviations:

| | |
|----------------|--|
| PBS | Phosphate Buffered saline |
| RSV | Resveratrol |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| HMG-CoA | 3-hydroxy-3- methylglutaryl-coenzyme A |

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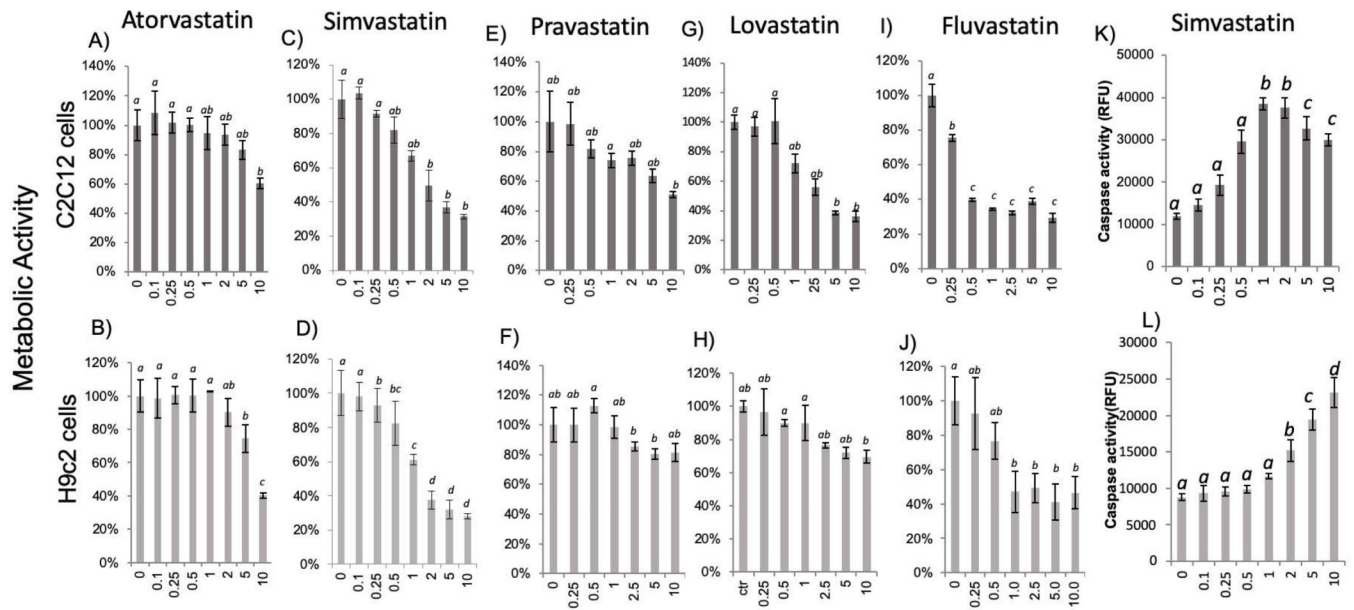


Fig 1. Statin Dose Dependent Inhibition of Metabolic Activity and Activation of Caspase. C2C12 cells (top panel) or H9c2 cells (bottom panel) were seeded in 24-well plates and were incubated with statins at indicated dose upon 80% confluency for 24 hours before measurements of metabolic activity using MTT assay (A-J) or for 16 hours for measurements of caspase activity using DEVD-AMC as a substrate (K, L). The results are from one experiment representative of three and are expressed as means \pm standard deviation (SD) from triplicates. Statistical significance ($p < 0.05$) was determined by ANNOVA and indicated by an alphabetic letter. The means labeled with “a” is significantly different from that labeled with “b”, “c” or “d”, where as “ab” indicates no significant difference from “a” or “b”.

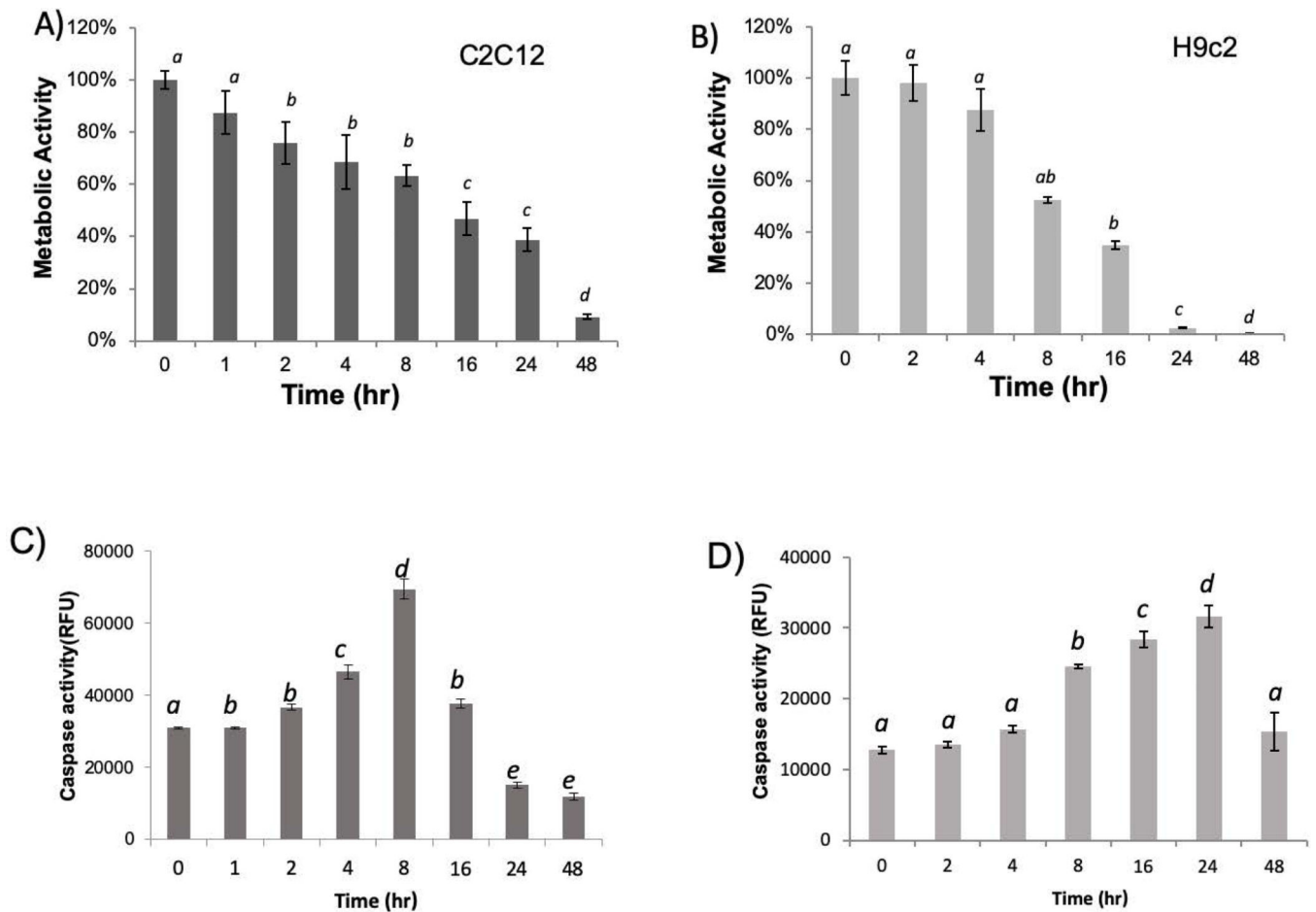


Fig 2. Time Dependent Inhibition of Metabolic Activity and Activation of Caspase.

C2C12 cells or H9C2 cells were seeded in 24-well plates. When cells reached 80% confluency, simvastatin was added to cells to 10 μ M in a reversed time scale, so that the metabolic activity can be measured by MTT assay at the same time (A, B), and caspase activity can be measured at the same time using DEVD-AMC as the substrate (C, D). The results are from one experiment representative of three and are expressed as means \pm standard deviation (SD) from triplicates. Statistical significance is indicated as described in Fig 1.

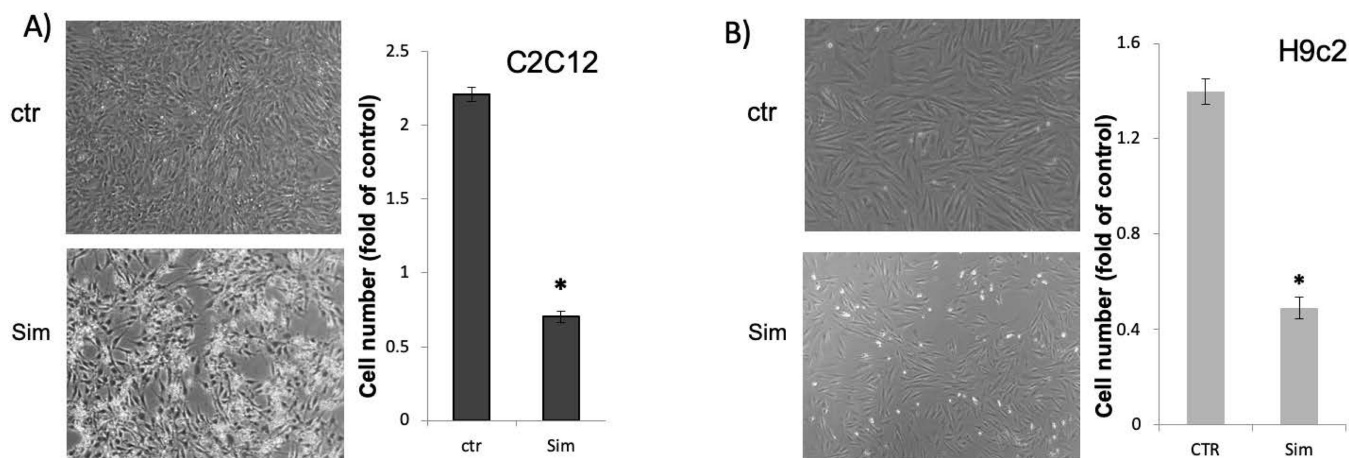


Fig 3. Loss of Cell Number due to Statin Treatment.

C2C12 cells or H9c2 cells were seeded in 6-well plates. When cells reached 60–80% confluency, simvastatin was added to cells to 10 μ M. Cell morphology was from untreated control versus 10 mM simvastatin treated cells at 24 hours after under an inverted microscope with 4x lens. Cell numbers of control or treated group over that at 24 hour before the treatment were recorded for counting and expressed as the percentage. 100% means no change in cell number, whereas percentage above 100% indicates cell proliferation, and less than 100% indicates loss of cells. The results are from one experiment representative of three and are expressed as means \pm standard deviation (SD) from triplicates. An asterisk indicates significant difference ($P < 0.01$) by student's t-test.

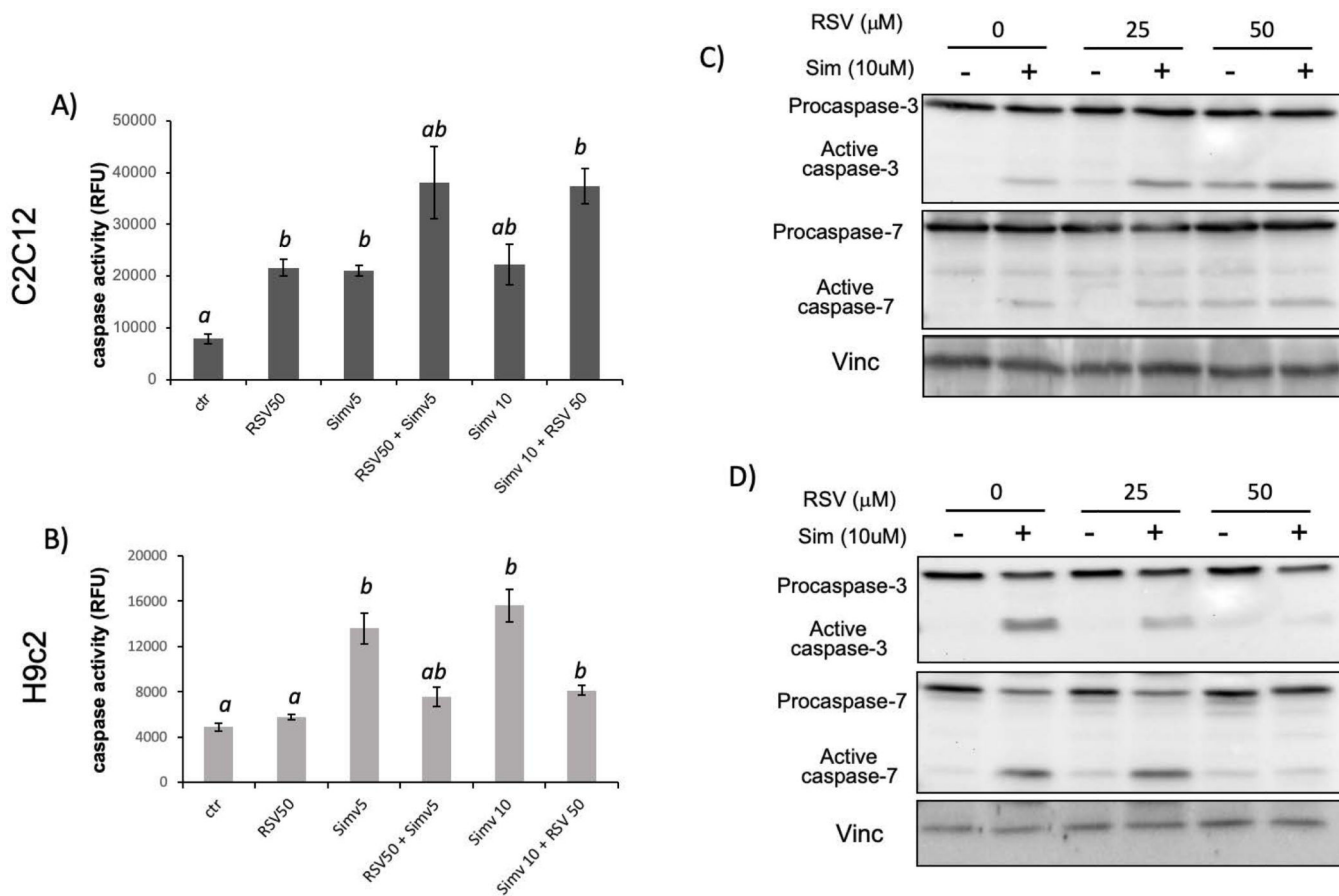


Fig 4. Resveratrol Inhibits Simvastatin from Activating Caspase-3 and Caspase-7. C2C12 cells or H9c2 cells were seeded in 24-well plates or 100 mm dishes. When cells reached 80% confluency, resveratrol was added to cells 1 hour prior to simvastatin treatment. Cells were harvested at 16 hours after for measurements of caspase activity using DEVD-AMC as a substrate or for Western blot to measure procaspase-3, procaspase-7 and active forms. The results are from one experiment representative of three and are expressed as means \pm standard deviation (SD) from triplicates (A, B). Statistical significance is indicated as described in Fig 1.

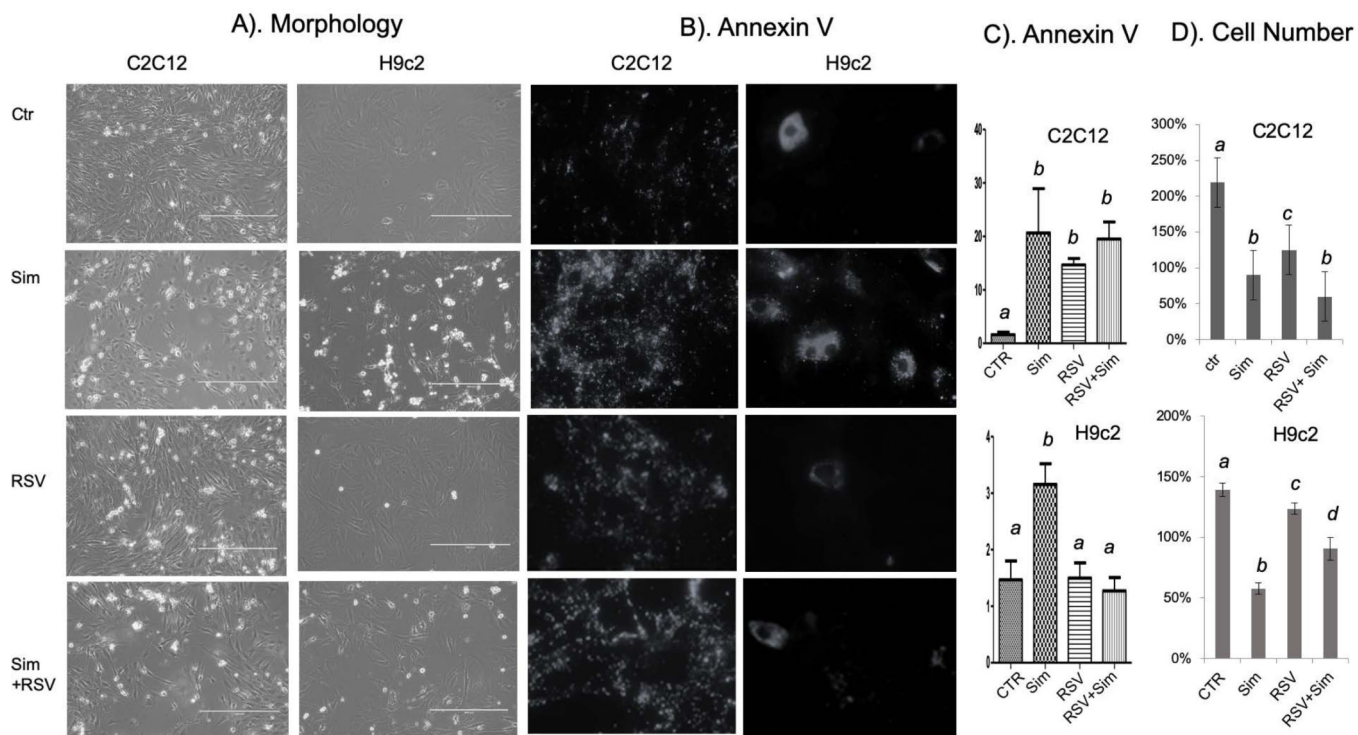


Fig 5. Morphological Evidence and Annexin V Staining Support that Resveratrol Prevents Simvastatin from Inducing Apoptosis.

C2C12 cells or H9C2 cells were seeded in 24-well plates containing coverglass or 6-well plates. When cells reached 60–80% confluency, resveratrol was added to cells 1 hour prior to simvastatin treatment. Morphology was recorded under an inverted microscope with 4xlens. Cells on coverglasses were stained with Annexin V for recording images under a fluorescence microscope and Annexin V positive cells were scored under a view of 20x lens. At least 4 views were scored for each treatment group for counting Annexin V positive cells (C). Cell number was counted prior to drug treatment and at 24 hours after drug treatment. Cell number was calculated as percentage over the number prior to drug treatment (D). The results are from one experiment representative of three (A, B), means \pm standard error (SE) from counting at least 4 views per group (C) or means + standard deviation (SD) from triplicates of one representative experiment. Statistical significance is indicated as described in Fig 1.